

**studies on
bone induction**

Klas Buring
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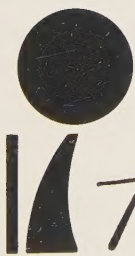
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 To Helena

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PREFACE

This thesis is a summary of the following communications referred to by their Roman numerals:

- I The Bone Induction Principle
Clin. Orthop. 53:243—283, 1967
In collaboration with Marshall R. Urist, Barry F. Silverman, Francois L. Dubuc and Joel M. Rosenberg.
- II Transfilter Bone Induction
Clin. Orthop. 54:235—242, 1967
In collaboration with Marshall R. Urist.
- III Effects of Ionizing Radiation on the Bone Induction Principle in the Matrix of Bone Implants
Clin. Orthop. 55:225—234, 1967
In collaboration with Marshall R. Urist.
- IV Osteogenic Competence
Clin. Orthop. 64:194—220, 1969
In collaboration with Marshall R. Urist, Phillip H. Hay, Francois Dubuc.
- V Ionizing Radiation for Sterilization of Bone
In: Sterilization and Preservation of Biological Tissues by Ionizing Radiation, International Atomic Energy Agency, Vienna, 1970.
- VI On the Origin of Cells in Heterotopic Bone Formation
Clin. Orthop., in press.
- VII Enzyme Patterns during Bone Induction
Acta orthop. scand. (Submitted, June 5, 1974.)

INTRODUCTION

The term *induction* was coined by Spemann in 1912 (Review, Spemann 1936)⁶⁰. The idea that embryonic cells respond to signals of their immediate environment was new and provided the starting point of an intense scientific effort to elucidate the nature of inductor systems. Numerous embryonic inductor systems have since been described⁷⁷ including skeletal tissues^{35, 36, 51}. Holtfreter (1933)³³ found that induction was a property residing not only in living tissues, but also in devitalized tissue or in tissue extracts. This finding led to the hypothesis that the message transmitted by the inductor must be spelled out in chemical terms and an intense search for its chemical identity started. A large number of substances and physical stimuli were suggested. According to a theory by Waddington (1966)⁷⁶, the inductor may be present in the competent tissue itself but bound in an inactive form. Thus, the inductor could be liberated by cytolysis and subsequently act upon other competent cells³⁴ causing an auto-induction.

The evidence for extracellular inductors gained further support by the experiments of Grobstein^{26, 27}, who used interposing filters with varying pore size. It has also been demonstrated that diffusible substances are liberated into the culture medium from mesodermal cells^{45, 59}. More recently it has been proposed that the mechanism of induction may operate by selective destruction of the cells of competent tissue².

It is generally assumed that inductors act by repressing or depressing genes during development and thereby initiate the sequence of processes commonly referred to as differentiation³¹. Despite the information hitherto available, it is still unknown how the inductor acts on the competent cell. Whatever the mechanism, it seems likely that activator substances may be far more universal than generally assumed, not only during embryological development but also in repair and healing processes in the adult mammal⁴². The range of action appears to be limited to the immediate cell microenvironment and also requires a certain

time and concentration of inductor substances. Functional modulations of target cells are well known to occur by the action of factors transported by the blood stream *i. e.* hormones. The possible effects of such substances on cell differentiation in tissue healing is an important field for further research.

A hypothesis — suggested by Bullough (1965)¹⁹ — that different tissues synthesize specific inhibitors (chalones) regulating the proliferation of their cells has opened up new vistas to a better understanding of mechanisms controlling the homeostasis of tissues. It thus appears that in many systems there is an interplay between stimulating substances and inhibitors to maintain an equilibrium. Whether such substances also take part in the regulation of bone formation is still an open question.

The literature on *bone transplantation* is abundant³⁰. (Reviewed by Chase & Herndon²², Burwell²¹ and Bassett^{11, 12, 13, 14, 15}). It concerns mainly transplantation into orthotopic sites, which makes it difficult to decide whether the new bone formation is caused by conduction from host bone or proliferation by transplanted bone cells. Heterotopic bone formation⁵⁵ (reviewed by Ostrowski & Wlodarski⁴⁷ is of greater interest in this context, as is induction by implants of devitalized skeletal tissues (reviewed by Bridges & Pritchard¹⁸).

According to certain workers¹⁷ bone extracts give rise to osteogenesis (Levander 1938⁴⁰, Annersten 1940¹, Levander & Willstaedt 1946⁴¹, Lacroix 1947³⁹). Others have been unable to verify these findings and stated that this may be seen even when non-specific solvents such as alcohol is injected. As stressed by Moss⁴⁴, this occurs normally only in periskeletal tissue and is rare in a true heterotopic area. Selle & Urist (1961)⁵², who studied bone formation caused by non-specific injury and inflammation, felt that other stimuli were necessary to induce regular bone formation in muscle. The reported incidence of positive bone formation in these experiments was generally low and unpredictable. However, in 1965 van de Putte & Urist⁴⁸ published a paper on osteogenesis in the interior of intra-muscular implants of decalcified bone matrix and reported regular yields of new bone. Urist (1965)⁶⁶ further more proposed a *theory of autoinduction* since both *inducing* and *responding* cells originated from the host, irrespective of whether the transplant was obtained from the same or another individual. The quantity of new bone formed was proportional to the available mass of inductively active matrix⁶⁷.

The use of demineralized bone transplants, however, was by no means new. Senn in 1889⁵³ advocated their use as a filling of cavities resulting from excisional surgery of osteomyelitis. Reports by other authorities of that time were conflicting and the method was rarely used. In more recent years Sharrard & Collins (1961)⁵⁴ reported its use in a few cases of spinal fusion in scoliotic children. The clinical use of decalcified bone transplants is at the present state not a realistic alternative to autogenous cancellous grafts. The ultimate test would be to provide evidence of new bone formation by decalcified matrix in a heterotopic area in man. So far this has not been demonstrated.

BACKGROUND AND AIM OF THE PRESENT STUDIES

The reader is referred to a comprehensive review by Urist⁷¹ of the work performed until 1970 at the Bone Research Laboratory, UCLA, Los Angeles. Further efforts at this laboratory to elucidate the nature of the inductor are continuously being published. Some of this work will be discussed in a later section^{23, 37, 38, 46, 61, 62, 63, 68, 69, 70, 72, 73, 74, 75}. Bone induction from demineralized dentin was the topic of a recent thesis by Dr. Gisle Bang of the Department of Pathology, Bergen, Norway. In a series of articles^{7, 8, 9, 10} he has focused attention on factors leading to a standardized experimental model in the guinea pig, the antigenicity of the matrix⁴ effects of proteolytic enzymes⁵, and effects of physical treatment⁶.

I had the privilege of acquiring a post-doctoral research fellowship in 1966—1967 and joined the research group of Dr. Marshall R. Urist at a time when the concept of the bone induction principle was formulated. The work initiated there has been continued and deals with bone induction from decalcified bone matrix implanted heterotopically. This is an experimental tool (i) firstly to evaluate the effects of various chemical and physical alterations of the organic matrix before implantation (I, III and V), (ii) secondly to study the cellular reactions of the host with morphological, autoradiographical and histochemical techniques (I, II, IV and VII).

EXPERIMENTAL MODEL

The basic experimental model throughout the present investigations was briefly as follows:

- (i) Diaphyseal bone from animals of the same species have been collected and cleaned from soft tissue and marrow. Bone cylinders with a length of 1—2 centimetres were cut and demineralized immediately or after temporary storage in a deep-freezer.
- (ii) The specimens were demineralized in dilute hydrochloric acid (HCl) at +4°C for the shortest possible time until decalcified as checked by X-ray. For rabbit bone this took 48 hours with one change of the acid. The matrix pieces were then washed in distilled water, frozen to -70°C, and lyophilized overnight. The specimen were stored in a dessicator at room temperature or polyurethane bags in a refrigerator at +4°C.

- (iii) Sterilization was usually not required provided specimens were handled under aseptic conditions. It could, however, be done in ethylene oxide gas and probably improves the incidence of positive bone induction.
- (iv) Experimental animals in these studies were mainly rabbits, but occasionally rats.
- (v) Through a midline incision 3—4 pouches on each side of the abdominal muscle were prepared by blunt dissection. One piece of demineralized, lyophilized bone matrix was introduced into each pouch.
- (vi) The implantation time was generally 4 to 6 weeks. At that time the abdominal plate was excised and fixed in formalin. X-rays of the excised muscle plate gave a rough estimate of the amount of new bone formed, if any.

The implantation sites were located in transillumination, and excised. Routine histological sections were prepared.

METHODOLOGICAL DISCUSSION

- (i) The bone has to be taken from the same animal (autogenic) or from animals of the same species (allogenic). Bone implants derived from animals of another species (xenogenic) did not result in new bone formation by induction but caused a heavy foreign body reaction despite the fact they were devitalized (I). Allogenic matrix also contains tissue antigens⁴ which, however, seems rather to enhance the induction process of the first set of implants. The second set, however, results in reduction of the inductive capacity and accelerated skin graft reaction.
- (ii) Undemineralized implants do not evoke bone induction or give at best on occasion small deposits of new bone. Crucial factors of demineralization are the type of acid or chelating agent, pH, time, and temperature^{6, 9, 61, 70}. Degradation of the inductive capacity takes place at neutral pH provided the temperature is not lowered and/or enzyme inhibitors (*e. g.* iodoacetic acid)⁷⁰ are added to the media. Evidence has been produced that this is due to the action of an endogenous enzyme, presumably a neutral peptidase which hypothetically has been termed bone morphogenetic proteinase (BMP-ase)³⁸. This explains the importance of treatment and storage at low temperature. If longer storage is needed, vacuum-sealed tubes should be preferred.

Deamination of the matrix prohibits induction and thus nitric acid cannot be used (I)⁴⁸. Formic and citric acid takes too long and may not lower the pH enough to eliminate the effect of endogenous proteinase. In contrast with our earlier findings chelating agents, such as EDTA, may be used, provided the temperature is kept at +2°C and enzyme inhibitors (IAA) are added⁷⁰.

- (iii) Sterilization by autoclaving (I), gamma irradiation (III, V), and beta-propiolactone treatment deteriorated the bone morphogenetic protein (BMP). The author has however demonstrated, that ethylene oxide gas does not interfere with the inductive capacity²⁰. This is corroborated by findings in demineralized dentin.
- (iv) In the laboratory it has proved necessary to use small rodents such as mice, rats, guinea pigs and rabbits owing to the large number of experiments. These rodents all give rise to bone induction from demineralized bone or dentin, however, special precautions are essential for mice⁷² and guinea pig⁹ matrices. My attempts to induce bone with decalcified allogenic matrix have so far failed in dog and man (females over 70). This may be due to lack of inductive capacity in dog and human bone, deterioration of the presumed bone morphogenetic protein during preparation, or a lack in competence (IV) of the host cells at the heterotopic implantation sites. Further work is needed to shed light on these mechanisms. Recent reports by Urist^{68, 78}, demonstrating great variation between species regarding the stability of bone morphogenetic properties during chemical extraction suggests that this problem will be solved in the future.
- (v) The implantation in the abdominal muscle is the crucial factor in this model as it constitutes a *heterotopic* implantation in contrast to the *orthotopic* implantation where the matrix is introduced into or at a pre-existing bone. In the latter case it is difficult to evaluate whether the new bone is formed by conduction from the host bed or due to induction by the implant.
- (vi) This experimental model serves as a bioassay of the inductive capacity of demineralized bone implants.

I. THE BONE INDUCTION PRINCIPLE

A series of experiments are reported concerning: (i) the sequence of events leading to bone induction, (ii) the cell populations during various intervals, (iii) phagocytosis, (iv) radioisotope uptake, (v) histocompatibility antigens, (vi) particle shape and size, (vii) chemical composition of the implant before and after induction, (viii) freezing and freeze-drying, (ix) heat, (x) lipid extraction, (xi) enzymatic degradation, (xii) collagen extraction, (xiii) organic blocking reagents. Bone induction from xenogenic matrices such as dentin, cartilage, fracture callus, muscle, tendon, and epithelium are discussed. The concept of a *bone induction principle* is suggested. The reason for choosing the term "principle" is that its nature is unknown and may be physical or chemical or both.

A modification of this working hypothesis was formulated by Urist (1970)⁶⁹ who suggested the more specific term *bone morphogenetic protein* (BMP), firmly bound to collagen but not part of it. Further a hypothetical endogenous neutral proteinase (BMP-ase)³⁸, with an effect similar to trypsin has been proposed and comprises the basis of his present work⁷⁵.

A thorough study of the antigenicity of allogenic dentin has recently been performed by Bang⁴. The effect of collagenase and trypsin on demineralized dentin matrix⁵ was investigated. An enhanced resorption of collagenase treated matrix was found whereas the trypsin treated implants decreased the phagocytic activity. In neither case, however, did any bone induction occur. It was suggested that the bone inducing capacity may reside within non-collagenous proteins of the matrix.

II. TRANSFILTER BONE INDUCTION

1. Cartilage, chondro-osteoid, or bone induction occurs in the interior of Millipore chambers loaded with minced muscle cells mixed with lyophilized decalcified bone matrix.

2. The mesenchymal cells induced to differentiate into bone on the inner wall produces transfilter bone induction of mesenchymal cells along the outer wall of the millipore membrane.

3. Neither the time nor the site of action or the physical-chemical properties of the bone induction principle is known, but the competent responding cell appears to be newly hypertrophied mesenchymal cell derived from proliferating endomysium.

4. Fractures, defects and leaks in the Millipore membrane that admit cells favour bone induction by direct contact between the mesenchymal cells and old matrix and impede the development of transfilter bone induction. The yield of transfilter bone induction is relatively scanty, while the yield of bone induction by matrix-mesenchymal cell coaptation is relatively great.

An impressive contribution to the existence of diffusible bone inductors from living cells of osteogenic origin has been given by Heiple et al.³². Working with Millipore chambers loaded with isogenous mouse osteosarcoma they were able to demonstrate formation of normal bone outside the chamber. This was not to be found in chambers containing the human osteosarcoma. The amount of bone produced outside of the chamber in the mouse was considerably increased compared with that of chambers containing normal mouse calvarias. In a further study this group examined the filters with the electron microscope²⁵ and concluded that cell-to-cell contact across the filter was unlikely. It was suggested that collagen or a filamentous material associated with collagen was related to the bone induction principle. Still, it is puzzling that a pore size of 0.1 micron inhibits induction, whereas 0.45 micron filters do not. Neither filter would reasonably hinder the diffusion of even very large macromolecules. This is discussed by Slavkin⁵⁶, and may be explained by the "budding-off" of RNA containing matrix vesicles described during epithelial-mesenchymal interaction of odontogenesis^{57, 58}.

III. EFFECTS OF IONIZING RADIATION ON THE BONE INDUCTION PRINCIPLE IN THE MATRIX OF BONE IMPLANTS

Samples of decalcified lyophilized bone matrix were exposed to graduated doses of gamma rays of ⁶⁰Co source and implanted into muscle pouches in the anterior abdominal wall of rats and rabbits. The dose of ionizing radiation currently used by bone banks for sterilization of bone tissue destroyed the bone induction principle. A smaller dose, in the range of 0.2 to 0.5 million r, may have disengaged the bone induction principle and enhanced bone induction, insofar as it produced a larger volume of new bone at an earlier interval of time than obtained from unirradiated samples of either autologous or isogeneic implants in extrasketal sites.

The effect of ionizing radiation on demineralized dentin⁶ has since been found to be in agreement with that reported in the present paper, apart from some differences probably due to the collagen of dentin being more stable than that of bone.

IV. OSTEOGENETIC COMPETENCE

Mesenchymal cell populations in connective tissues of thyroid, liver, spleen, kidney parenchyma, adrenal, migrate into the implant, proliferate, but resorb bone matrix only very slowly and fail to secrete an osteogenetic substratum.

Dual transplants of decalcified bone matrix and mesenchymal cell populations derived from muscle produce bone in liver, spleen or kidney parenchyma, organs devoid of cells with osteogenetic competence. Thus in post-fetal life differentiation of bone occurs not from blood-borne cells or vascular tissues but from a competent mesenchymal cell population proliferating in contiguity with a substratum of extracellular substances prepared from pre-existing calcified tissue.

Mesenchymal cells migrate from a free strip of autologous muscle, display a selective affinity for the surface of an implant of allogeneic decalcified bone matrix, proliferate and differentiate into new bone in host beds in liver, spleen, or kidney parenchyma. Mesenchymal cell populations display different levels of readiness to differentiate into bone in an implant of bone matrix. Measured in terms of percentages of positive results and yields of new bone, the levels are: bone and bone marrow > skeletal muscle > subcutaneous tissue > dermis > brain > lung > anterior chamber of eye > testes > pancreas > ovary. Mesenchymal cells are differentiated cell populations, not equally competent or omnipotent, and display strictly prescribed programs for further development following transplantation. One program included osteogenetic competence, an inactive or stored state of readiness to differentiate into bone.

V. JONIZING RADIATION FOR STERILIZATION OF BONE

With recent evidence that osteogenic inductors are present and active in the organic matrix of allogeneic bone implants it is clearly vital to know what effect treatment and storage may have upon this inductive capacity. Ionizing radiation from linear accelerators or ^{60}Co sources assures sterility in a dose of 2—4 Mrad but possible effects on the organic matrix have deserved less attention. The present experimental investigation has shown changes from three different points of view. (1) Biological: The inductive capacity of decalcified, lyophilized allogeneic rat and rabbit implants was totally destroyed at a dose of 2 Mrad. The implants either evoked a heavy foreign body reaction or were totally resorbed. (2) Chemical: The solubility of collagen and glycosaminoglycans was investigated by neutral and weak acid extractions. It was found to increase continuously with a marked rise at 2 Mrad. The sub-units of collagen were determined by electrophoresis and chromatography, the findings suggesting scission of covalent bonds in the alpha chain as well as dearrangement of intra- and intermolecular helical structure. (3) Ultrastructural: The regular fibrillar network of bone matrix was destroyed by doses exceeding 2 Mrad.

A definite negative effect of radiation sterilization of dry-bone matrix has thus been demonstrated. Biological inactivity of the implant with an increase in its solubility, fast resorption, a foreign-body reaction and total disruption of

the ultrastructural pattern. The alterations demonstrated in this experiment strongly suggest, that a careful appraisal regarding the possible negative effect of gamma-sterilization should be made when biological material is irradiated.

VI. ON THE ORIGIN OF CELLS IN HETEROTOPIC BONE FORMATION

Previous experiments have demonstrated decalcified, lyophilized bone matrix to be a powerful inductive substrate for differentiation of mesenchymal cells into new bone.

The present experiment employed parabiosis as a tool to differentiate cells of haematogenous origin from cells originating from the mesenchyme at the site of implantation of bone matrix. The results indicate that *the inductor relasing cells* are blood-borne monocytoïd cells which enter the tissues by diapédésis and which are transformed into histiocytes, macrophages, matrixclasts and osteoclasts. It is suggested that their precursors are located in bone marrow at sites remote from the area of bone induction. In contrast, *the responding cells* of this system which develop into osteoblasts and osteocytes are derived from local perivascular mesenchymal retaining their competence to from bone.

VII. ENZYME PATTERNS DURING BONE INDUCTION

Bone morphogenesis by implants of bone matrix in muscle in rabbits has been the experimental tool to study enzyme patterns during the induction of new bone formation.

Histochemistry of frozen sections from various time intervals after implantation revealed a general pattern for the appearance of various oxidative and hydrolytic enzymes.

Succinic dehydrogenase and beta-glucuronidase are particularly characteristic of apparently blood-borne monocytoïd cells present in the early time interval when resorption of the matrix is initiated. Macrophages and multinuclear matrixclasts increase and display activity in particular for acid phosphatase and succinic dehydrogenase. Only after this matrix resorption is under way does alkaline phosphatase appear in a different celltype presumably local fibroblasts, indicating enzyme induction. An increased mitotic activity among these cells brings about a rapid increase in alkaline phosphatase and eventually a change in cellular type giving rise to osteoblasts and new bone formation.

Lactic dehydrogenase isoenzyme shift indicates increased vascularity at the same time as the resorption process is initiated.

Quantitative analysis on tissue homogenates for alkaline phosphatase, acid phosphatase and lactic dehydrogenase corroborates the histological observations.

GENERAL DISCUSSION

Demineralization of bone with hydrochloric (HCl) or ethylenediaminetetraacetic acid (EDTA) leaves a residue containing about 88 % collagen and 6 % non-collagenous protein. After implantation in muscle this matrix gives rise to cell migration, proliferation and differentiation with the formation of new bone. It also has a morphogenetic capacity in that a spherical ossicle is produced with central bone marrow. Except that resorption of the matrix precedes differentiation, the mechanism by which the matrix acts is unknown. Small amounts of chondro-osteoid may be induced *in vitro* or in diffusion chambers provided the matrix is placed in a mixed mesenchymal cell population. The inducing capacity of the matrix is susceptible to a large number of physical and chemical agents as well as to degradation by a tentative endogenous neutral proteinase.

There is now evidence indicating that the cells responsible for the resorption of the matrix are blood-borne monocytoïd cells, migrating into the implantation area as wandering histiocytes, and displaying specific enzyme characteristics. It is proposed that their predecessors are located at remote sites in bone marrow, in contrast with the bone forming osteoblasts.

The mechanisms for initiating the resorption, for stimulating the migration of macrophage cells, for fusion of these cells, for modulation of their enzyme activity, for giving the mitotic stimulus to the proposed stem-cells in remote bone-marrow are all unknown.

The resorption apparently results in the release of an inductor residing in the matrix. The responding fibroid cells show, firstly, a change in functional state^{49, 65} as evidenced by an increase in alkaline phosphatase and, second after mitrotic divisions, a change in cell type to preosteoblasts, osteoblasts and osteocytes.

Several theories of bone differentiation prevails assume electrical¹⁶, mechanical¹⁶, or chemical factors of more or less specific type^{42, 71}. Recently these theories have been considered and a common cause has been suggested²⁹. Assuming that the BMP bone morphogenetic protein is positively charged there should be an accumulation at sites with a local surplus of negative charge which is known to induce mesenchymal cells to differentiate into osteoblasts.

Osteogenesis and osteoclasia have three different fundamental prerequisites, namely the proper cell, the proper nutrition, and the proper stimulus. The latter two constitute the microenvironment of the cell¹⁶. As different progenitors have been established for osteoclasts and osteoblasts one might wonder whether there may not be separate inducing systems for osteogenesis and osteoclasia.

At present two concepts prevail as to the mechanisms in embryologic induction^{24, 64}.

- (i) a short initial inductive stimuli by specific macromolecules triggers the whole pathway,^{43, 50, 77} or
- (ii) only a first step in a long chain of events is released, and this is followed by secondary interactions between tissue components²⁸.

Antigen processing by macrophage cells³ may provide an analogue to induction. If so, matrix-resorbing cells would take up a proposed BMP by endocytosis and synthesize a RNA moiety, afterwards complexed with the inductor. This hypothetical RNA-BMP complex may then enter responding cells. The RNA part is here linked to non-inductor-specific sites of the DNA of the fibroblast. This in turn would lead to differentiation and new synthesis of bone.

Future work on bone induction must be concentrated not only on the characterization of the inductor, but also on the intracellular control of biosynthesis.

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